REMARKS

Reconsideration is requested.

Claims 7 and 11 have been additionally canceled, without prejudice, to advance prosecution. Claims 7 and 11-14 are pending. Claims 12-14 will be pending upon entry of the present Amendment.

A response to the Request of May 11, 2009 is requested along with a return of an initialed copy of the PTO 1449 Form filed January 23, 2009.

To the extent not made moot by the above amendments, the Section 103 of claims 7 and 11-14 over Ikhlef (U.S. Patent Application Publication No. 2003/0064374) and Dalton (WO 95/11887), is traversed. Reconsideration and withdrawal of the rejection are requested in view of the following distinguishing comments and the attached.

The rejection is understood to be based on an assumption that treating Alzheimer's disease (AD) would necessarily cause improvements in the symptoms of AD, namely cognitive deficits. The applicants submit, with due respect, that this assumption is in error.

AD is a progressive neurodegenerative disorder that is characterized by the presence of amyloid deposition and neurofibrillary tangles together with the loss of cortical neurons and synapses (see e.g., Ritchie, K. & Lovestone, S. The dementias. Lancet 360, 1759–1766 (2002); Terry, R.D. et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann. Neurol. 30, 572–580 (1991)). Abnormalities have also been reported in peripheral

tissue supporting the fact that Alzheimer's disease is a systemic disorder (Connolly, G., Fibroblast models of neurological disorders: fluorescence measurement studies TiPS Vol. 19, 171-77 (1998)).

Besides cognitive deficits, AD symptoms include, for instance, behavioural disorganization, disability to walk, incontinence, psychiatric complications or metabolic problems. Accordingly, treating AD may designate addressing any one of the above symptoms, not necessarily perceptive cognition. A substantial number of drugs presently used for treating AD are anti-depressants and anti-psychotics, which have no effect on perceptive cognition.

Examples of anti-depressants used in AD include the following serotonin-reuptake inhibitors:

- citalopram (Celexa)
- fluoxetine (Prozac)
- paroxeine (Paxil)
- sertraline (Zoloft)
- trazodone (Desyrel)

Suitable anxiolytics include benzodiazepine agonists such as :

- lorazepam (Ativan)
- oxazepam (Serax)

Antipsychotic medications include dopamine D2 receptors, serotonin 5-HT receptors, alpha-1A-adrenoceptors, histamine H1 receptors:

- aripiprazole (Abilify)

- clozapine (Clozaril)
- haloperidol (Haldol)
- olanzapine (Zyprexa)
- quetiapine (Seroquel)
- risperidone (Risperdal)
- ziprasidone (Geodon)

The mention in Ikhlef et al that etazolate can be used to treat AD thus does not imply or suggest an effect on perceptive cognition.

The results presented in the present application are unexpected because, for example, they rely on a novel mechanism of action of etazolate, not previously foreseen or predicted by the cited art, namely the modulation of GABAA receptor causing sAPPalpha production. As mentioned in the application, the inventors have discovered that GABA is involved in cognition and that etazolate, in addition to being a PDE4 inhibitor, is also a GABAA receptor modulator. These results have been further disclosed by the inventors in Marcade et al (JNC, 106:392-404 (2008) "Etazolate, a neuroprotective drug linking $GABA_A$ receptor pharmacology to amyloid precursor protein processing"). This publication shows that, by eliciting GABA mechanisms, etazolate further protects against Aβ peptide and induces the precognitive sAPPα molecule. This dual mode of action of Etazolate is unprecedented.

The remarkable properties of etazolate have led the assignee to initiate clinical trials with this molecule for increasing perceptive cognition in AD patients. Phase I trials have now been completed and a Phase IIa trial conducted on 197 ambulatory patients

suffering from mild to moderate Alzheimer's disease is about to be finalized. As stated in the attached recent Press Release of the company (wherein etazolate is designated EHT0202) (emphasis added):

> EHT 0202 has a **novel mechanism** of action when compared to existing Alzheimer's disease therapeutics: it stimulates the α-secretase pathway, thus enhancing the production of the **procognitive** and neuroprotective sAPPα fragment of APP (Amyloid Precursor Protein). ...
> Phase I studies demonstrated good tolerability of EHT 0202 in both young and aged healthy volunteers; importantly, no sedation was observed clinically.
> Preclinical studies have shown that EHT 0202 protects cortical neurons against Aβ42-induced stress and that this neuroprotection is associated with sAPPα induction. EHT 0202 has also **demonstrated pro-cognitive properties in several animal models**: age-related memory impairment and scopolamine-induced amnesia (2). ...
> "In light of the neuroprotective **and procognitive** effects demonstrated by EHT 0202 in preclinical studies, we believe that our drug candidate has the potential to modify the course of Alzheimer's disease and might open a new era in the treatment of this devastating disease"

The novel mechanism and pro-cognitive effect of etazolate were unexpected and unpredictable from the cited art. The claimed methods would not have been obvious in view of the cited combination of art.

Withdrawal of the Section 103 rejection is requested.

The Examiner is requested to confirm consideration of the attached art pursuant to MPEP § 609.05(c).

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. The Examiner is requested to contact the undersigned, preferably by telephone, in the event anything further is required in this regard.

Respectfully submitted,

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Etazolate, a neuroprotective drug linking $GABA_A$ receptor pharmacology to amyloid precursor protein processing

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Abstract

Pharmacological modulation of the $GABA_A$ receptor has gained increasing attention as a potential treatment for central processes affected in Alzheimer disease (AD), including neuronal survival and cognition. The proteolytic cleavage of the amyloid precursor protein (APP) through the α-secretase pathway decreases in AD, concurrent with cognitive impairment. This APP cleavage occurs within the β-amyloid peptide (Aβ) sequence, precluding formation of amyloidogenic peptides and leading to the release of the soluble N-terminal APP fragment (sAPPα) which is neurotrophic and procognitive. In this study, we show that at nanomolar-low micromolar concentrations, etazolate, a selective $GABA_A$ receptor modulator, stimulates sAPPα production in rat cortical neurons and in guinea pig brains. Etazolate (20 nM–2 μM) dose-dependently protected rat cortical neurons against Aβ-induced toxicity. The neuroprotective effects of etazolate were fully blocked by $GABA_A$ receptor antagonists indicating that this neuroprotection was due to $GABA_A$ receptor signalling. Baclofen, a $GABA_B$ receptor agonist failed to inhibit the Aβ-induced neuronal death. Furthermore, both pharmacological α-secretase pathway inhibition and sAPPα immunoneutralization approaches prevented etazolate neuroprotection against Aβ, indicating that etazolate exerts its neuroprotective effect via sAPPα induction. Our findings therefore indicate a relationship between $GABA_A$ receptor signalling, the α-secretase pathway and neuroprotection, documenting a new therapeutic approach for AD treatment.

Keywords: alpha secretase, Alzheimer's disease, amyloid β protein, etazolate, GABA, soluble APP ectodomain.

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Alzheimer's disease (AD) is characterized neuropathologically by the extracellular deposition of the 4 kDa β-amyloid peptide (Aβ), a 39–43 amino acid peptide that is neurotoxic (Borchelt *et al.* 1996) and accumulates in neuritic plaques and in cerebral and meningeal microvessels (Wahrle *et al.* 2002). Amyloid precursor protein (APP) is the precursor of Aβ and can be processed via alternative pathways. A non-amyloidogenic secretory pathway includes cleavage of APP to soluble APP (sAPPα) by α-secretases from the a disintegrin and metalloprotease (ADAM) family of proteases within the Aβ peptide sequence, thus precluding the formation of Aβ. In contrast, the formation of the amyloidogenic Aβ peptides is regulated by the sequential action of β- and γ- secretases (Checler 1995; Nunan and Small 2000). A primary strategy proposed to treat AD is to prevent the formation of Aβ peptides, and their deposition as senile plaques in the brain. Recently, Postina *et al.* (2004) showed that activation of α-secretase significantly reduces AD-like pathology in an animal model of AD. This raises the possibility that a pharmacological approach to increase α-secretase activity and sAPPα levels, and thus reduce Aβ formation may be suitable for AD treatment (Bandyopadhyay *et al.* 2007).

This approach is consistent with the observation that profound changes occur in APP processing with aging and the development of AD. In particular, a reduction in the activity of the non-amyloidogenic pathway leading to

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Abbreviations used: Aβ, beta amyloid; ADAM, a disintegrin and metalloprotease; AD, Alzheimer's Disease; APP, amyloid precursor protein; BACE, beta-site APP-cleaving enzyme; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester; IP, immunoprecipitation; LTP, long-term potentiation; PDE4, phosphodiesterase 4; PTX, picrotoxin; sAPPα soluble APP ectodomain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAPI-1, TNF-α Protease Inhibitor-1.

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decreased sAPPα levels both in brain and in the periphery are thought to play a role in the deficits of CNS functions associated with AD and normal aging (Lannfelt *et al.* 1995; Nistor *et al.* 2007). Likewise, previous studies have demonstrated that sAPPα stimulates long-term potentiation (LTP), which is a candidate cellular mechanism for learning and memory, has potent memory-enhancing effects (Meziane *et al.* 1998; Ring *et al.* 2007) and plays a role in the promotion of neurite outgrowth, synaptogenesis and synaptic plasticity (Turner *et al.* 2003). In particular, sAPPα and α-secretase ADAM10 overexpression increase cholinergic, glutamatergic and GABAergic cortical synaptogenesis *in vivo* (Bell *et al.* 2006a). Moreover, sAPPα exerts anti-apoptotic and neuroprotective effects against excitotoxic and oxidative insults, and has been shown to prevent Aβ-induced neurodegeneration *in vitro* and *in vivo* (Mattson *et al.* 1993). It has been suggested that these effects may also rely on the ability of sAPPα to recruit, amplify or differentiate stem cells or progenitors (Caille *et al.* 2004; Chen *et al.* 2006). Therefore, shifting the processing of APP towards the neurotrophic α-secretase pathway may not only reduce the formation of senile plaques, slowing down the evolution of the disease, but may also result in symptomatic improvement.

APP processing and regulation of the α-secretase activity are under control of several major neurotransmitters (Ulus and Wurtman 1997) and are dependent upon neuronal electrical and synaptic activity (Nitsch *et al.* 1993; Kamenetz *et al.* 2003; Farber *et al.* 1995; Marcello *et al.* 2007). Cortical cholinergic innervation also promotes the α-secretase pathway and the release of sAPPα (Nitsch *et al.* 1992). In addition, sAPPα release occurs after the *in vivo* induction of LTP in the dentate gyrus (Fazeli *et al.* 1994). Therefore, modulating neuronal excitability could positively impact APP processing and prove beneficial in AD.

The amino acid γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the human brain. GABA is a key signaling factor that controls LTP (Dawson *et al.* 2006). GABA also has well-known trophic actions on central nervous system development (Mohler 2007) and controls the various steps in adult neurogenesis (Bordey 2007). Beyond its acknowledged role in cognition and epilepsy, GABA's association with brain diseases and neuropsychiatric symptoms and disorders (see Lanctot *et al.* 2004 for review) make it a likely therapeutic target for the treatment of dementia.

Multiple studies provide evidence supporting GABAergic dysfunction in AD (Lanctot *et al.* 2004). Studies using transgenic models of early-stage amyloid pathology have suggested that amyloid pathology progresses in a neurotransmitter-specific manner where cholinergic terminals appear most vulnerable, followed by glutamatergic terminals and finally by GABAergic terminals (Bell *et al.* 2006b). Further strengthening the potential benefit of GABA in AD, a number of recent studies have associated $GABA_A$ receptor signalling with significant neuroprotection against Aβ–mediated toxicity (Gu *et al.* 2003; Louzada *et al.* 2004; Lin and Jun-Tian 2004, Lee *et al.* 2005). These results suggest that selective pharmacological modulation of $GABA_A$ receptors could offer a disease modifying approach in AD in addition to providing symptomatic benefit.

Drugs that interact at the $GABA_A$ receptor can possess a spectrum of pharmacological activities, depending on their ability to modify the actions of GABA. For example, $GABA_A$ receptor agonists such as diazepam enhance the inhibitory effects of GABA at the $GABA_A$ receptor and show effects that translate into, for example, the anxiolytic, sedative, and amnesia promoting properties observed clinically. By contrast, non-selective inverse agonists show opposing effects and could be beneficial clinically in terms of enhancing cognition. However, those molecules exhibit anxiogenic and convulsant/proconvulsant activities that prevent their use in humans. Clearly, a selective compound possessing a modulatory effect at the $GABA_A$ receptor capable of eliciting the neuroprotective and cognition-enhancing effects and devoid of sedative, anxiogenic and convulsant/proconvulsant properties would be of clinical utility for AD.

Etazolate is a pyrazolopyridine compound that belongs to a family of molecules with anxiolytic-like properties (Patel *et al.* 1985) which selectively modulates the $GABA_A$ receptor (Barnes *et al.* 1983; Whiting *et al.* 1997; Thompson *et al.* 2002). The results presented in the present study demonstrate that etazolate exerts a neuroprotective effect against Aβ via the $GABA_A$ receptor. This neuroprotection is associated with sAPPα induction via the α-secretase pathway. Our findings represent the first pharmacological link between $GABA_A$ receptor signalling, neuroprotection against Aβ, and stimulation of the α-secretase pathway and indicate that modulation of the $GABA_A$ receptor pathway can offer both symptomatic and disease modifying therapeutic potential for the treatment of AD.

Experimental procedures

Materials and reagents

The following antibodies were used: 3E9 (mouse monoclonal antibody, Stressgen); 6E10 (mouse monoclonal antibody, Signet); anti-APP (Cter) AHP538 antibody (rabbit polyclonal antibody, Serotec); cytochrome c antibody (mouse monoclonal antibody, Calbiochem). Control mouse IgG was from Tebu. Etazolate (1-ethyl-4-(N'-isopropylidene-hydrazino)-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid ethyl ester hydrochloride) was synthesized at Panchim (Lisses, France). Muscimol, baclofen, picrotoxin, Gabazine/SR95531, and bicuculine were obtained from Sigma. TNF-α Protease Inhibitor-1 (TAPI-1), Furin Inhibitor I, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT),

beta-site APP-cleaving enzyme (BACE) inhibitor II, and BACE fluorogenic substrate were purchased at Calbiochem. Recombinant sAPPα, Aβ42 and Aβ25–35 were from Biosource and Sigma. All cell culture reagents were from Invitrogen (Cergy Pontoise, France) unless otherwise noted. Mouse/rat Aβ40 ELISA assay kit was from IBL (Hamburg, Germany).

Primary cell cultures and treatments

Cortical cells were isolated from 17 days old Wistar rat embryos (Janvier). Cells were dissociated by trituration using 0.25% trypsin. Cells were plated in 6 μg/mL polyornithine-coated plates at 500 000 cells per cm^2 in medium (Neurobasal, 1X B27, 2 mM L-glutamine, 0.6% glucose, and 1X mix antibiotic and antimycotic from Sigma) containing 2% horse serum. Cells were maintained at 37°C in a humidified 5% CO2 incubator. The day after plating, cells were treated with 5 μM of antimitotic Cytosine arabinofuranoside (AraC). After 4 days *in vitro*, one half of the medium was changed with medium without horse serum. The culture was allowed to mature in this medium which was changed twice a week for 7–10 days.

Fibrillar Aβ42 was generated according to Isaacs *et al.* (2006) and preparations were analysed on SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and Western blot. At the beginning of each experiment, 7–10 days old neuronal cultures were changed and treated with the indicated concentration of etazolate or with 50 pM recombinant human sAPPα. After 6 h incubation time with etazolate, Aβ42 or Aβ25–35 peptide preparations were added to the cultures at the concentration of 7.5 μM or 33.5 μM, respectively. At these concentrations, both peptides generated 40–50% cell death after 48 h incubation. Muscimol and baclofen were incubated 24 h before Aβ addition (Lee *et al.* 2005). Picrotoxin, Gabazine/SR95531, bicuculine, TAPI and Furin inhibitor I were pre-incubated 1 h before etazolate addition at 50 μM, 20 μM, 10 μM, 10 μM and 30 μM, respectively. For sAPPα neutralisation assay, 3E9 antibody or control IgG (5 μg/mL each) were added to cortical cell cultures concomitantly to etazolate or sAPPα treatment, followed by Aβ42 or Aβ25–53 peptides as described above. After 48 h incubation, cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

In vitro sAPPα western blot analyses

Cortical neurons grown in 6 well plates were scraped and lysed in CelLytic-M (Sigma). Protein concentrations were determined by the Bradford procedure. Equal protein quantities were separated on a 10% SDS-PAGE gel and transferred to Hybond-C (Amersham Biosciences) membranes. After transfer, membranes were blocked with 5% non-fat milk and incubated overnight with the indicated primary antibody. For sAPPα detection, cells were allowed to secrete for 24 h. Media were collected, cleared by centrifugation, and then equal amounts were loaded on 10% SDS-PAGE and Western blotted with 3E9 monoclonal antibody (1/500). Immunological complexes were revealed with an anti-mouse peroxidase-conjugated antibody (Jakson Laboratories, 1/15 000) followed by ECL enhanced chemiluminescence (Amersham Biosciences). The relative levels of sAPPα, APP, and Cytochrome c were determined by densitometry of the scanned images by using Photoshop software (Adobe).

Soluble sAPPα immunoprecipitation

In all, 20 ng of recombinant sAPPα (IBL, Japan) were prepared in 250 μL sample buffer (EIA buffer) of the Human sAPPα Assay Kit (IBL) with a protease inhibitor mixture (Roche Applied Science) and incubated with or without 5 μg anti-sAPPα 3E9 antibody overnight under rotation at 4°C. The sAPPα-antibody complexes were precipitated using 50 μL of washed/equilibrated protein G beads in EIA buffer containing the protease inhibitor mixture for 4 h under rotation at 4°C to allow complexes to bind to protein G on the EZview Red Protein G affinity Gel beads (Sigma). The samples were centrifuged for 10 min at 8.200 *g* at 4°C. The supernatants were assayed for sAPPα by sandwich ELISA according to the manufacturer's instructions (IBL).

Cytotoxicity assay

Cell viability was assessed in 96 well plates using MTT assay. Mann-Whitney U test and Wilcoxon test were used to determine the significance between the data means. Significance values are as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ versus corresponding control.

Measurement of cytochrome *c* release

The anti-Cytochrome *c* antibody used here is a mouse monoclonal (isotype IgG2b) that reacts with denatured human, mouse, and rat cytochrome *c*. Cortical neurons grown in 6 well plates were trypsinized and centrifuged at 1000 *g* for 5 min. The pellet was washed in ice-cold phosphate buffered saline and centrifuged again at 1000 *g* for 5 min. The cells were resuspended with 1X Cytosol Extraction Buffer containing dithiothreitol and Protease inhibitors, incubated on ice for 10 min, and homogenized in an ice-cold tissue grinder (30–50 passes). The homogenate was centrifuged at 700 *g* for 10 min at 4°C and the supernatant was centrifuged at 10 000 *g* for 30 min at 4°C. The resulting supernatant was loaded (30 μg protein/lane) on a 10% SDS-PAGE gel, probed with cytochrome *c* antibody at 1/200 according to standard Western Blot procedures.

sAPPα and APP dosages in Guinea pigs

Etazolate or vehicle (physiological saline) were administered in male Hartley albino guinea pigs, weighing 250–270 g at delivery and obtained from Charles River Laboratories (L'Arbresle, France), once a day for 15 consecutive days by per os route at 10 mg/kg. One hour after the final administration, the guinea pigs were killed. Brains were immediately extracted and immersed in an oxygenated (95% O2, 5%CO2) physiological saline bath placed on ice (1–2°C), and superficial vessels were removed. The whole brains were dissected to provide left and right cortices, which were weighted, snap-frozen in liquid nitrogen, and separately stored at −80°C. The maximum time between sacrifice and snap freezing was less than 15 min. Left cortices were homogenized at 4°C in 4 vol/weight of 20 mM Tris pH 7.5 containing 0.2% Triton X-100, 50 μg/mL gentamycin and a protease inhibitor mixture (Roche Applied Science) and centrifuged at 20 000 *g* for 6 h at 4°C. Supernatants were normalized to total protein using BCA, and assayed for soluble sAPPα by sandwich ELISA according to the manufacturer's instructions (IBL Human sAPPα Assay Kit). Mann-Whitney U test and Wilcoxon test were used to determine the significance between the data means. Significance values are as follows: $****p < 0.0001$ versus vehicle control. APP levels were determined in these same

fractions by loading 10 μg protein/lane on a 4–12% SDS-PAGE gel probed with the primary anti-APP antibody that recognizes the C terminal part of APP and not sAPPα, used at 1/1000 dilution.

Measurements of Aβ40 levels

Cortical neurons cultured in 24 well plates were incubated for 24 h in the presence of various concentrations of etazolate, the beta secretase inhibitor (BACE inhibitor II, Z-Val-Leu-Leu-CHO) or the gamma-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl-Lalanyl)]-S-phenylglycine t-butyl ester). Media were collected, centrifuged and assayed for rat Aβ40 by sandwich ELISA according to the manufacturer's instructions (IBL).

Beta-secretase and gamma-secretase assays

The beta secretase and gamma-secretase assays (Désiré *et al.* 2005) were performed on cortical neurons cellular extracts after 24 h treatment of cells with etazolate, the beta secretase inhibitor or the gamma-secretase inhibitor. For the beta secretase assay, total proteins were extracted in 20 mM 2-(N-morpholine)-ethane sulfonic acid (MES), 1% Triton X-100 plus protease inhibitor mixture by incubation on ice for 30 min. The assay was carried out in a volume of 200 μL of reaction buffer (25 mM MES, 25 mM sodium acetate, 25 mM Tris, pH 4.4), containing 25 μL of the preparation plus 15 μM peptide Substrate V (Calbiochem), a quenched fluorogenic substrate containing the Swedish mutation MCA-SEVNLDA-EFK(DNP)-CONH2. Excitation was performed at 320 nm and the reaction kinetics were monitored by measuring the fluorescence emission at 420 nm on a Fluoroscan Ascent FL plate reader (Thermo LabSystems). For the γ-secretase activity assay, solubilized γ-secretase fractions were generated from membrane preparations incubated in ice-cold CelLytic-M (Sigma) during 15 min at 4°C on a shaker. Cell debris and nuclei were removed by centrifugation at 1000 *g* for 15 min. at 4°C, membrane preparations were pelleted at 20 000 *g* for 1 h at 4°C and resuspended in 100 μL of activity buffer (150 mM sodium citrate, pH 6.4). Solubilized γ-secretase activity was induced at 37°C for 2 h and Aβ40 generated *de novo* was quantified using the rat Aβ40 ELISA.

Results

Effect of etazolate on sAPPα secretion in neuronal cortical cell cultures

The effect of the $GABA_A$ modulator etazolate on sAPPα release was analyzed in rat neuronal cortical cell culture supernatants. By using an N-terminal monoclonal antibody against APP, 3E9 (Hoppens Gylys *et al.* 2004), we showed by Western Blot that rat sAPPα migrated as a single band at the expected molecular mass (Fig. 1). Induction of secreted sAPPα was dose-dependent and Fig. 1(a) shows a representative gel depicting an increase in sAPPα secretion with increasing concentrations of etazolate after a 24 h treatment. sAPPα induction occurred as early as 4 h (data not shown). The levels of secreted sAPPα after treatment with nanomolar and micromolar concentrations of etazolate reached a plateau, and maximal sAPPα level was obtained at 2 μM etazolate and was about 2.9-fold above its basal level

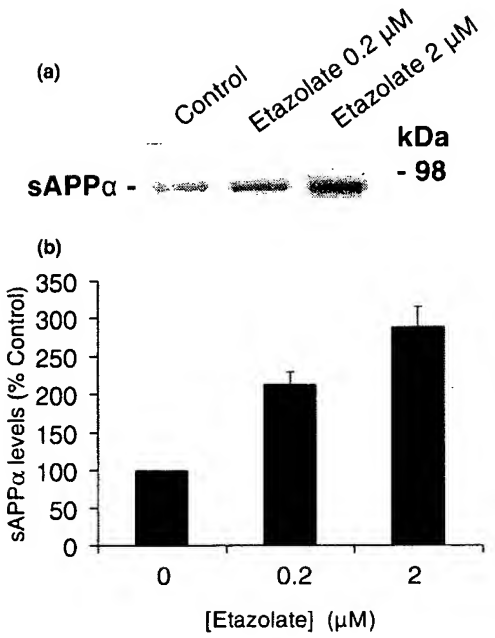

Fig. 1 The $GABA_A$ modulator etazolate dose-dependently increases secreted levels of non-amyloidogenic sAPPα in cortical neurons. (a) Representative immunoblot showing the effect of increasing concentrations of etazolate on the cellular release of sAPPα from rat cortical neurons. After 24 h incubation, the culture medium was collected and sAPPα was measured by western blot using the 3E9 antibody. (b) Quantitation of specific sAPPα released for cortical neurons. Results are means ± SEM of three independent experiments performed in duplicate and are expressed as the percentage of control untreated cells in the same experiment.

(Fig. 1b). At concentrations as low as 0.2 μM, etazolate generated a two fold induction over basal level. At higher concentrations (i.e. 10 μM), no further increase was evidenced (data not shown).

Etazolate induces sAPPα production *in vivo*

Etazolate was tested in guinea pigs to determine whether the observed stimulation in sAPPα release in cortical cell cultures can be reproduced *in vivo*. We used normal wild-type albino guinea pigs as a model, because they are an established model for closely mimicking physiological APP processing and sAPPα production (Beck *et al.* 2003; Désiré *et al.* 2005). In addition, sequence identity between human and guinea pig sAPPα allowed the use of a sandwich human sAPPα ELISA.

Preliminary experiments performed in rats showed that etazolate overall displays good tolerability and brain penetrance (data not shown). Therefore, we delivered etazolate *per os* over 15 days at the concentration of 10 mg/kg, a concentration ensuring brain exposure compatible with the pharmacologically active doses defined *in vitro* for sAPPα release (our unpublished observations). Levels of sAPPα

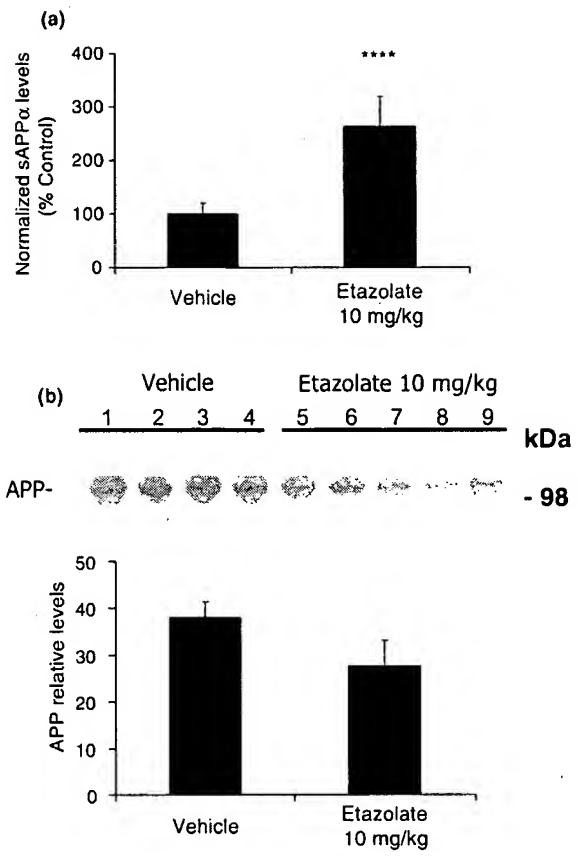

Fig. 2 Etazolate increases brain sAPPα in Guinea pigs. (a) sAPPα levels in cortex after administration of vehicle or etazolate at 10 mg/kg (daily p.o. administration for 15 consecutive days). Results are expressed as the percentage of control animals. ****$p < 0.0001$ versus vehicle by Wilcoxon test. (b) APP levels detected by western Blot in vehicle- or etazolate-treated animals and corresponding quantitation.

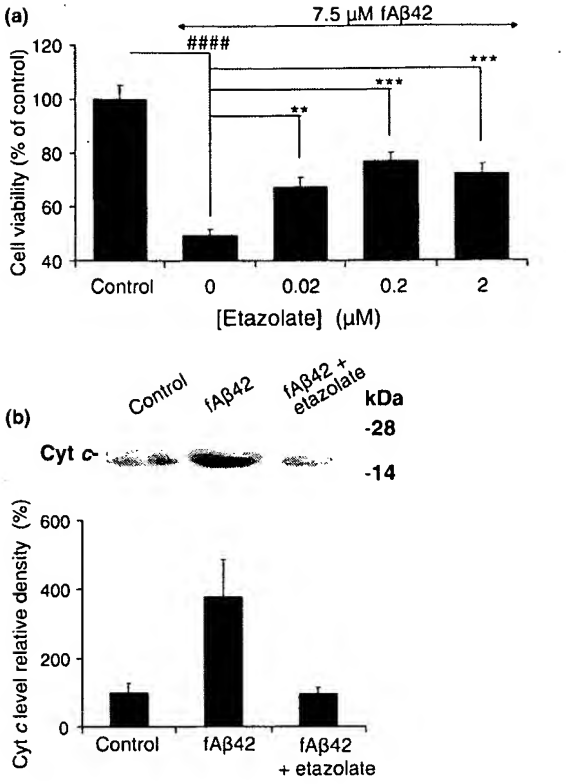

Fig. 3 Etazolate dose-dependently prevents the neurotoxicity of fibrillar Aβ42 on cortical neurons. (a) Neuronal viability determined by MTT assay. Results are expressed as percentages of control cultures and correspond to means ± SEM of twelve independent experiments. ####$p < 0.0001$ compared to control; **$p < 0.01$; ***$p < 0.001$ versus 0 μM etazolate. (b) Etazolate blocked Aβ42-induced release of cytochrome *c* from mitochondria in cortical neurons. Cortical neurons were treated with 7.5 μM of fAβ42 for 48 h with or without 6 h pre-incubation with etazolate (0.2 μM). After treatment, cytochrome *c* release was analyzed by immunoblotting. An immunoblot of cytochrome c representative of three independent experiments is shown.

were measured in the soluble cortical fractions (Fig. 2a). In control animals, recovered sAPPα concentration per 100 μg total protein was 0.74 ± 0.15 ng/mL. Etazolate increased brain sAPPα almost three times with $p < 0.0001$ (by Wilcoxon test) as compared to vehicle control animals. Individual analysis of all animals indicated that all animals responded to etazolate treatment. Full-length APP levels were analysed by western blot in etazolate- and vehicle-treated animals (Fig. 2b). The anti-APP antibody used here recognizes the C terminal part of APP and not sAPPα. Quantitation of the western blots indicated a 30% decrease in APP levels in etazolate–treated animals, suggesting that more APP may be cleaved via the α-secretase pathway.

Neuroprotective effect of etazolate on fibrillar Aβ42-induced toxicity in cortical cell cultures

Aβ peptide induces neurodegeneration of cortical and hippocampal neurons through oxidative stress, secondary excitotoxicity and a wide range of molecular events that disturb neuronal homeostasis. The $GABA_A$ receptor has been implicated in the neuroprotection against Aβ (Gu *et al.* 2003; Louzada *et al.* 2004; Lin and Jun-Tian 2004; Lee *et al.* 2005). Therefore, the effect of etazolate on cell viability was examined in cortical neurons treated with fibrillar Aβ42 peptide preparations (fAβ42). By itself, etazolate (0.2 μM–100 μM) does not affect viability of cortical neurons or any other neuronal cell type tested (data not shown). In these cortical neurons, 7.5 μM fAβ42 induced around 40–50% toxicity after 48 h incubation ($p < 0.0001$ by Wilcoxon test). A single addition of etazolate 6 h before fAβ42 was added to the cells very significantly inhibited fAβ42-induced toxicity, as visualised after a 48 h incubation (Fig. 3a). For the following experiments, the concentration of 0.2 μM for

etazolate was used to assess the protective effects against fAβ42-induced neuronal cell damage.

Aβ42-induced cell death is accompanied by caspases activation (Chong *et al.* 2006). The release of cytochrome *c* from mitochondria, an upstream signaling component of caspases activation, was therefore investigated. The release of cytochrome *c* was induced by fAβ42 after 24 h incubation on cells. Etazolate (0.2 μM) pre-incubated 6 h before fAβ42 treatment significantly inhibited Aβ42-induced cytochrome *c* release (Fig. 3b).

Neuroprotective effect of etazolate against different Aβ peptides is $GABA_A$ dependent

Etazolate is selective for the $GABA_A$ receptor and does not bind to the $GABA_B$ receptor. We initiated a first set of experiments to implicate $GABA_A$ signalling for neuroprotection against Aβ, using either a $GABA_A$ (muscimol) or a $GABA_B$ (baclofen) receptor agonist, used at the concentration of 5 μM (Louzada *et al.* 2004; Lee *et al.* 2005). Muscimol protected cortical neurons by 43.2 ± 9.6% while Baclofen was ineffective in this Aβ toxicity model (Fig. 4a). Neuroprotection by muscimol was in the same range than previously published results on these neuronal cultures. These data indicated that in cortical neurons, $GABA_A$ but not $GABA_B$ signaling is active for protection against Aβ42-induced toxicity.

Etazolate binding to the $GABA_A$ receptor can be blocked by bicuculine (Bic), an allosteric inhibitor of $GABA_A$ receptor opening (Barnes *et al.* 1983). Therefore, the potential neuroprotective properties of etazolate against

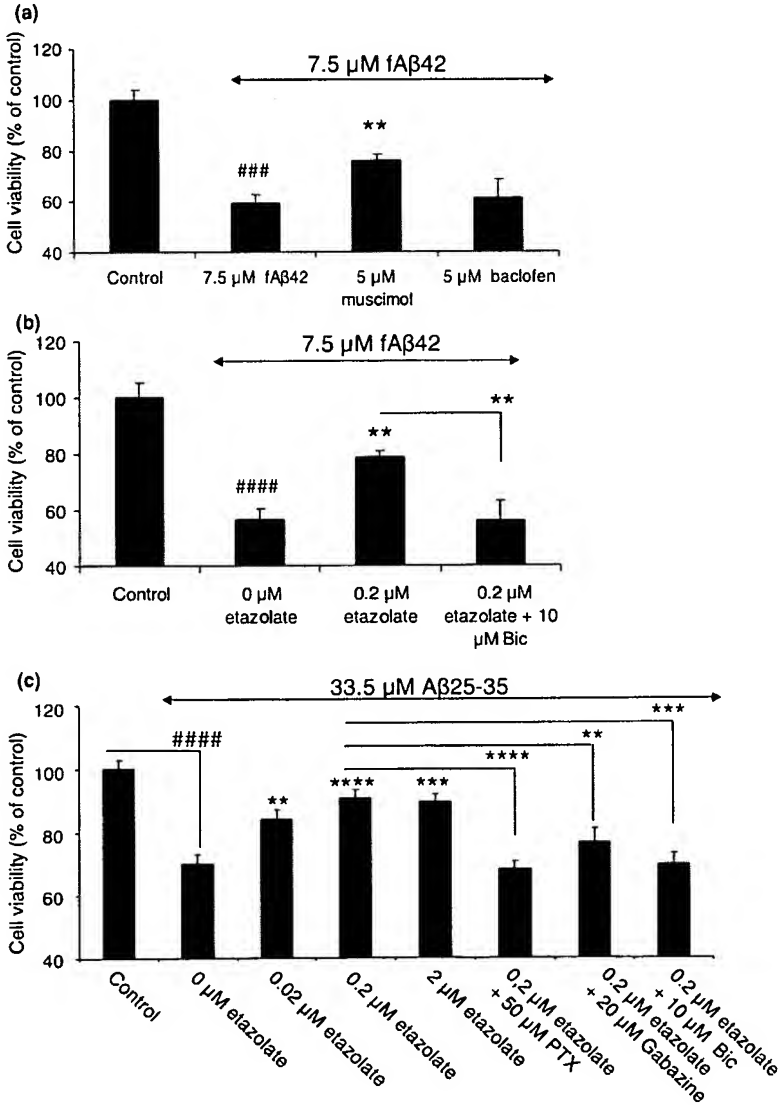

Fig. 4 Etazolate prevents the neurotoxicity of Aβ on cortical neurons via the $GABA_A$ receptor. (a) Cortical neurons were treated with 7.5 μM of fAβ42 for 48 h with or without pre-incubation with muscimol or baclofen. Neuronal viability was determined by MTT assay. Results are expressed as percentages of control cultures and correspond to means ± SEM of three independent experiments. ###$p < 0.001$ compared to control; muscimol treatment: **$p < 0.01$ by Wilcoxon test. (b) Etazolate neuroprotection against fAβ42 toxicity is prevented by $GABA_A$ receptor inhibitor bicuculine (Bic). ####$p < 0.0001$ compared to control; **$p < 0.01$ versus 0 μM etazolate. Etazolate plus Bic cotreatment: **$p < 0.01$ versus 0.2 μM etazolate by Wilcoxon test. (c) Aβ25–35 neurotoxicity is prevented by etazolate in a $GABA_A$-dependent manner. Cells were treated with etazolate with or without $GABA_A$ receptor inhibitors picrotoxin (PTX), Gabazine/SR95531, bicuculine (Bic) for 6 h at the indicated concentration and intoxicated with 33.5 μM Aβ25–35 for 48 h. Neuronal viability was determined by MTT assay. Results are expressed as percentages of control cultures and correspond to means ± SEM of seven independent experiments. ####$p < 0.0001$ compared to control; Etazolate treatment: **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ versus 0 μM etazolate; Etazolate plus $GABA_A$ receptor inhibitors cotreatment: **$p < 0.01$; ***$p < 0.001$ ****$p < 0.0001$ versus 0.2 μM etazolate by Wilcoxon test.

Aβ42 toxicity were investigated in the presence or absence of Bic (10 μM). At this concentration, Bic did not affect Aβ toxicity by itself (data not shown), as already shown by others (Louzada *et al.* 2004; Lee *et al.* 2005). Bic abolished the neuroprotective effect of etazolate upon fAβ42 treatment (Fig. 4b). Cell viability returned to the level of Aβ-treated cultures (cell viability (% of control) was: 56.5 ± 3.7 (fAβ42 alone), 78.5 ± 2.3 (fAβ42 + etazolate), 56.25 ± 6.7 (fAβ42 + etazolate + Bic), $p < 0.01$).

Then, we extended these results using Aβ25–35, a fragment of Aβ42 containing the aminoacids 25 to 35 that has been widely used to study the impact of $GABA_A$ pharmacology on Aβ-induced neurotoxicity in cortical neurons (see Lee *et al.* 2005). Aβ25–35 does not fully mimic Aβ42 neurotoxicity but retains the ability to impact LTP (Gu *et al.* 2003) and cognition (Klementiev *et al.* 2007). We treated cortical cell cultures with Aβ25–35 (33.5 μM) after a 6 h pre-incubation with etazolate. Aβ25–35 induced 30–40% toxicity and etazolate (0.02–2 μM) was able to significantly inhibit Aβ-induced toxicity. Cell viability (% of control) was 90.8 ± 2.6 for the concentration of 0.2 μM etazolate and this concentration was chosen for the following experiments.

As with fAβ42, etazolate effect on Aβ25–35-induced neuronal death was fully prevented by Bic pre-treatment (Fig. 4c). Bic, however, can have non-GABAergic effects via calcium-dependent potassium channels. Therefore, treatment by two other $GABA_A$ receptor antagonists, gabazine and picrotoxin (PTX), is pharmacologically necessary to assess whether etazolate acts through a $GABA_A$ receptor–dependent mechanism. PTX is a GABA chloride channel blocker, while Bic and Gabazine are allosteric inhibitors of $GABA_A$ receptor opening. As Bic, PTX has also been previously shown to prevent etazolate effect at the $GABA_A$ receptor (Barnes *et al.* 1983). PTX (50 μM) and Gabazine (20 μM) when pre-incubated at these subtoxic concentrations before etazolate addition (0.2 μM), abolished etazolate neuroprotective effect (Fig. 4c). In fact, cell viability returned to the level of Aβ-treated cultures for all three antagonists [cell viability (% of control) was: 70 ± 2.8 (Aβ25–35 alone), 90.8 ± 2.6 (Aβ25–35 + etazolate), 68.1 ± 4.5 (Aβ25–35 + etazolate + PTX), 76.5 ± 3.6 (Aβ25–35 + etazolate + Gabazine), 69.5 ± 4.3 (Bic)].

Overall, Aβ25–35 neurotoxicity was more reproducible than that of fAβ42 in our hands. Since we have clearly implicated the $GABA_A$ receptor in etazolate–mediated neuroprotection against both Aβ42 and Aβ25–35, all subsequent experiments were performed with Aβ25–35.

The neuroprotective effect of etazolate on Aβ-induced toxicity is associated with α-secretase activity

Disintegrin metalloproteases, including ADAM10 and ADAM17, catalyze the shedding of the ectodomain of APP which produces the neurotrophic sAPPα. We next investigated whether blocking α-secretase activity could impede etazolate-mediated neuroprotection against Aβ. To this aim, TAPI, a selective, competitive inhibitor of α-secretase ADAM17 was used. ADAM10 and ADAM17 require proteolytic processing to become active. The proprotein convertase furin has been linked to the proteolytic cleavage of adamalysins (Endres *et al.* 2003). Accordingly, the Furin Inhibitor CMK suppresses sAPPα release (Hwang *et al.* 2006). Therefore, we also investigated whether the Furin Inhibitor could prevent etazolate-mediated neuroprotection. Cortical neurons were pre-treated with TAPI (10 μM) or the Furin Inhibitor (30 μM) 1 h before etazolate (0.2 μM) addition. This protocol allowed strong reduction of basal and etazolate-stimulated sAPPα levels (Fig. 5a) while Aβ25–35 toxicity remained unaffected (Fig. 5b). Etazolate reduced Aβ25–35 toxicity by 41.9% and TAPI and the Furin Inhibitor blocked etazolate-induced neuroprotection against Aβ25–35. These findings indicated that neuroprotection against Aβ are dependent on a furin or a furin-like protease metalloprotease

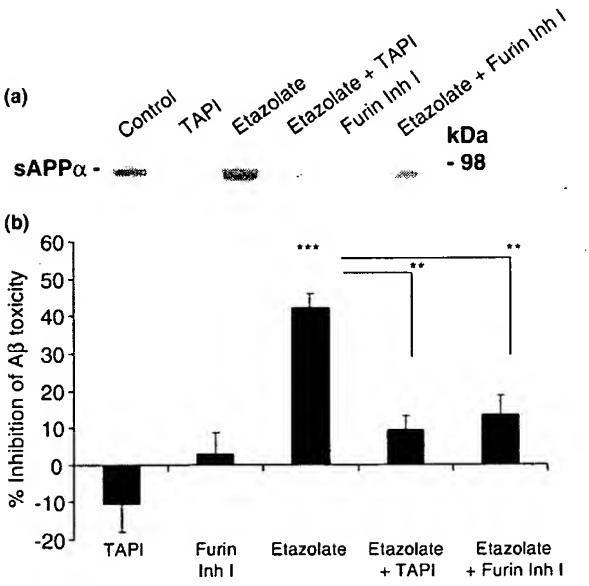

Fig. 5 sAPPα release stimulation and neuroprotection against Aβ25–35 by etazolate are associated with α-secretase activity. (a) Representative immunoblot showing the inhibitory effect of ADAM proteases inhibitors TAPI (10 μM) and the Furin Inhibitor 1 (30 μM) on etazolate (0.2 μM) – induced sAPPα production. (b) TAPI and the Furin Inhibitor 1 prevent etazolate-mediated neuroprotection against Aβ. Cells were treated with etazolate (0.2 μM) with or without pre-incubation with TAPI (10 μM) or the Furin Inhibitor 1 (30 μM) and intoxicated with 33.5 μM Aβ25–35. Neuronal viability was determined by MTT assay after 48 h incubation. Results are expressed as percentages of control cultures and correspond to mean % inhibition ± SEM of at least three independent experiments. Etazolate treatment: $^{***}p < 0.001$ versus 0 μM etazolate. Etazolate versus etazolate + TAPI or Furin Inhibitor I cotreatments: $^{**}p < 0.01$ by Wilcoxon test.

activity and that sAPPα producing ADAM proteases, including ADAM17, are likely involved.

The neuroprotective effect of etazolate on Aβ-induced toxicity is associated with sAPPα induction

To directly implicate sAPPα induction in the neurotrophic activity of etazolate, a sAPPα neutralization assay was implemented. Such strategy has previously been used to demonstrate the implication of sAPPα, in the proliferation of progenitors in the adult subventricular zone (Caille *et al.* 2004). The antibody commonly used for sAPPα neutralization, 22C11, could not be used here because it exhibited dose-dependent cytotoxicity on rat cortical cultures (data not shown). In fact, this antibody has been shown to participate to APP-mediated neuronal death (Rohn *et al.* 2000). 6E10, another described sAPPα neutralizing antibody, was not potent at neutralizing rat sAPPα, probably due to the three aminoacid residues difference that exist between the human and rat epitopes (data not shown). We therefore investigated a panel of different other antibodies. One of them, clone 3E9, is a monoclonal antibody that binds another region comprised between amino acids 18 and 38 of the APP N terminus which is similar in rat and human and is able to recognize rat sAPPα in Western Blot (see Fig. 1).

To demonstrate the sAPPα neutralization potential of this antibody, we used in preliminary experiments human recombinant sAPPα because of the 100% sequence identity between rat and human sAPPα around the epitope region. sAPPα was diluted at 80 ng/mL in phosphate buffered saline-Tween 20-bovine serum albumin and was subsequently immunoprecipitated using 3E9. After immunoprecipitation (IP), the resulting supernatant was analysed in a human specific sAPPα ELISA (Fig. 6a). The overall recovery of the ELISA assay determined in our conditions is close to 100% (data not shown). Immunoprecipitation by 3E9 antibody (IP) lead to a very strong decrease in the remaining quantity of sAPPα [sAPPα concentrations were: 83.7 ± 1.4 ng/mL (no IP) and 20.8 ± 0.2 ng/mL (IP)].

In a second step, we determined the neutralization potential of 3E9 antibody on recombinant sAPPα in a neuroprotection assay. Preliminary experiments indicated that optimal sAPPα concentration was 50 pM, allowing 51.1 ± 3.3% neuroprotection against Aβ after 48 h. Cortical neurons intoxicated with Aβ25–35 for 48 h were therefore incubated or not with 50 pM recombinant sAPPα in the presence or absence of a non-cytotoxic concentration of 3E9 antibody (5 μg/mL). As shown in Fig. 6b, 3E9 did not affect cell viability by itself. In the presence of Aβ, cell viability was reduced to 68 ± 1.6% as compared to control and increased to 84.9 ± 3.3% in the presence of sAPPα (51% inhibition of Aβ toxicity). In the presence of 5 μg/mL 3E9, sAPPα neuroprotection was abolished (cell viability was 68.2 ± 10.9%), which indicated that 3E9 is a neutralizing antibody for sAPPα. In contrast, a control mouse IgG (same

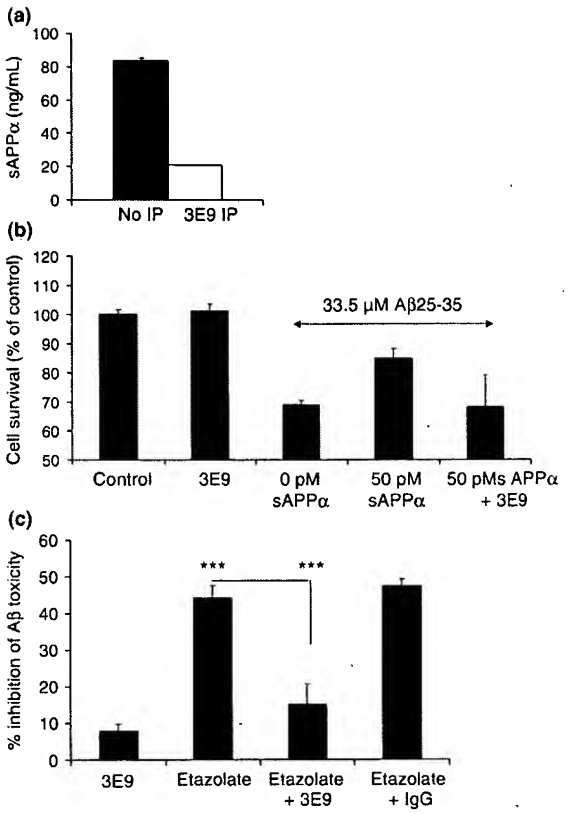

Fig. 6 Etazolate prevents the neurotoxicity of Aβ25–35 on cortical neurons via sAPPα production. (a) 3E9 monoclonal antibody immunoprecipitates sAPPα. Recombinant human sAPPα spiked at 80 ng/mL in phosphate buffered saline-Tween 20-bovine serum albumin was immunoprecipitated using 3E9 or not and the resulting supernatant was analysed on sAPPα specific ELISA. Note the strong depletion of sAPPα remaining after immunoprecipitation (IP). (b) 3E9 antibody prevents the neuroprotective effect of recombinant human sAPPα against Aβ25–35. Cells were treated with 50 pM sAPPα with or without 5 μg/mL 3E9 antibody and were intoxicated with 33.5 μM Aβ25–35. Neuronal viability was determined by MTT assay after 48 h incubation. Results are expressed as percentages of control cultures and correspond to mean % inhibition ± SEM of three independent experiments. (c) 3E9 antibody prevents the neuroprotective effect of etazolate against Aβ25–35. Cells were treated with etazolate (0.2 μM) with or without 5 μg/mL 3E9 antibody or a control IgG, and were intoxicated with 33.5 μM Aβ25–35. Neuronal viability was determined by MTT assay after 48 h incubation. Results are expressed as percentages of control cultures and correspond to mean % inhibition ± SEM of eighth independent experiments. Etazolate treatment: $^{***}p < 0.001$ versus 0 μM etazolate; Etazolate versus etazolate + 3E9: $^{***}p < 0.001$ by Wilcoxon test.

concentration) was not effective in preventing sAPPα–induced neuroprotection (data not shown). As shown in Fig. 6c, 3E9 did not affect Aβ toxicity. We then tested the effect of 3E9 on etazolate-induced sAPPα release and subsequent neuroprotection. Cortical neurons were intoxi-

cated with Aβ25–35 in the presence or absence of 0.2 μM etazolate with or without 3E9 antibody (5 μg/mL), and then after 48 h, cell viability was determined. As shown in Fig. 6c, etazolate reduced Aβ–induced toxicity by 44.3 ± 3.2% and its effect was prevented by 3E9 antibody (in the presence of 3E9 antibody, etazolate-induced inhibition of Aβ toxicity was 15.2 ± 5.5%). In contrast, the control mouse IgG (5 μg/mL) did not affect etazolate-mediated neuroprotection. Taken together, these data indicated that the neuroprotective properties of etazolate upon Aβ toxicity are associated to the production of sAPPα via the non-amyloidogenic α-secretase pathway in cortical neurons.

Etazolate does not impact the amyloidogenic pathway

To test whether etazolate altered the amyloidogenic pathway or not, we determined Aβ40 levels secreted from cortical neurons after 24 h incubation with etazolate. As shown in Fig. 7a, etazolate did not modify Aβ levels at the concentrations tested (0.2 and 2 μM). The α-secretase inhibitor TAPI (10 μM) did not significantly change Aβ levels, which were in contrast strongly reduced in contrast by the cell permeable BACE inhibitor II and the γ-secretase inhibitor DAPT used at the concentration of 10 μM.

We then tested the effect of etazolate on overall cellular β-secretase activity using a BACE fluorogenic assay in cell cultures treated with etazolate (0.2 μM) or BACE inhibitor II (10 μM). The assay is based on the cleavage of the quenched fluorogenic Substrate V, containing the Swedish mutation of APP, resulting in increased fluorescence (Andrau *et al.* 2003). The BACE inhibitor II abolished the cleavage, while incubation of cells with etazolate did not affect fluorescence as compared to control, indicating that etazolate has no effect on BACE activity (Fig. 7b). Then, we determined that etazolate is not inhibiting γ-secretase activity. We used a γ-secretase assay allowing *de novo* Aβ generation from APP, using cell membranes as the source of both γ-secretase and its substrate, the C99 fragment of APP (Pinnix *et al.* 2001; Désiré *et al.* 2005). γ-secretase activity was monitored by the Aβ40 ELISA and DAPT was included as control. Incubation of cells with 0.2 μM etazolate for 24 h did not result in changed *de novo* Aβ40 production while DAPT (10 μM) strongly reduced Aβ40 levels (Fig. 7c). Taken together, our data indicated that etazolate effect on the α-secretase pathway does not involve inhibition of the amyloidogenic pathway.

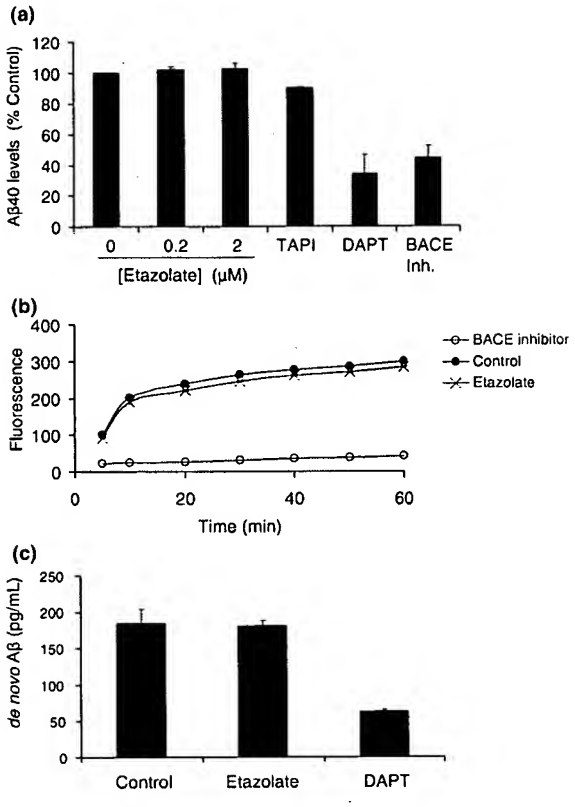

Fig. 7 Etazolate does not affect the amyloidogenic pathway. (a) Etazolate does not affect Aβ40 levels released from cortical neurons. Cortical neurons were treated with various doses of etazolate, or with TAPI, DAPT or BACE Inhibitor II (10 μM each) for 24 h and supernatants were processed using rat Aβ 40-specific ELISA to determine Aβ40 levels. Results shown represent the mean (± SEM) of three independent experiments. (b,c) Etazolate does not act as an inhibitor of β-secretase (b) or γ-secretase (c) activity in cortical neurons. (b) β-secretase activity (cleavage activity of β-secretase fluorogenic substrate) was determined by fluorescence recording in homogenate proteins from cortical neurons that were treated or not with various doses of etazolate for 24 h. Etazolate (0.2 μM) did not affect fluorescence while very low substrate cleavage activity was detected in cells treated with BACE inhibitor II (10 μM). Results shown represent the mean (± SEM) of three independent experiments. (c) γ-Secretase assay, as monitored using Aβ40-specific ELISA to quantitate *de novo* Aβ40 production by solubilized γ-secretase activity. Incubation of cortical neurons with etazolate (0.2 μM) for 24 h did not change γ-secretase activity, in contrast to treatment with γ-secretase inhibitor DAPT (10 μM). Results shown represent the mean (± SEM) of three independent experiments.

Discussion

This work reports the effects of etazolate, a $GABA_A$ receptor modulator, on the α-secretase pathway and the neuroprotection against Aβ-induced toxicity. Two major findings were identified: at pharmacological concentrations that can be reached *in vivo* (i.e. nM to low micromolar concentrations), etazolate (1) induces sAPPα through the stimulation of the α-secretase pathway, and (2) exerts a neuroprotective effect against Aβ which is associated with sAPPα induction. Taken together, our data suggest that etazolate's neuroprotective properties involve the stimulation of sAPPα production, providing a link between $GABA_A$ pharmacology and APP processing for neuroprotection.

A major finding of the present study is that etazolate neuroprotective activity is associated with sAPPα induction. Stimulating the α-secretase pathway is of interest for both neuroprotective and symptomatic AD therapy since sAPPα exerts anti-apoptotic and neuroprotective effects against excitotoxic, oxidative, and Aβ-related insults *in vitro* and *in vivo* (Postina *et al.* 2004, Mattson *et al.* 1993), in addition to being procognitive. Furthermore, sAPPα increases cholinergic, glutamatergic and GABAergic cortical synaptogenesis (Bell *et al.* 2006a). Therefore, etazolate, which modulates $GABA_A$ receptors, can further reinforce its synaptic impact through the production of sAPPα. Future efforts will further delineate the mechanisms linking GABA signalling and sAPPα. At least in neuroblastoma SH-SY5Y cells, using anti-ADAM-10 and anti-ADAM-17 antibodies, we found no evidence that etazolate acts to stimulate α-secretase proteins expression or maturation. However, the importance of APP trafficking within the cell, and its cleavage locations, with α-secretase on the plasma membrane/late secretory vesicles and β-secretase in endosomes and trans-Golgi network, make it possible that etazolate increases APP-α-secretase colocalization such that the non-amyloidogenic cleavage of APP via the α-secretase pathway is facilitated. Follow-up α-secretase activity assays will be performed to test this hypothesis. In support of this, increased sAPPα levels in guinea pig brains are associated with a slight (30%) reduction in total APP levels following etazolate treatment. This may indicate that more APP is cleaved through the stimulated α-secretase pathway.

A number of studies indicate that an increase in activity of α-secretases, particularly ADAM10, in the brain of AD patients may be achieved by modulating selective signaling pathways that increase their activity or expression level (Hashimoto *et al.* 2003; Robert *et al.* 2005; Kojro *et al.* 2006). For most of these molecules, the property of stimulating the α-secretase pathway might be related to their neuroprotective and procognitive properties. However, to our knowledge, none of these studies causally implicated sAPPα. Here, in contrast, we demonstrate that etazolate neuroprotective mechanism is associated with sAPPα induction because (1) etazolate dose-dependently induces sAPPα, (2) sAPPα mimics the neuroprotective effect of etazolate, and (3) sAPPα neutralization or the prevention of its production by various ADAM inhibitors prevents etazolate effects on Aβ-induced neuronal death. We quantified sAPPα release from cortical cultures using an indirect method of titration with known quantities of human recombinant sAPPα comigrating with rat sAPPα on the same western blots. In 24 h, sAPPα accumulates in conditioned medium from cortical cell cultures in the picomolar concentration range which is sufficient for neuroprotection (Stein *et al.* 2004).

We also show that etazolate potently inhibits both fibrillar Aβ42–induced neuronal death and neuronal death induced by its neurotoxic fragment Aβ25–35 in a $GABA_A$ dependent manner. Aβ42 is a major contributor to the pathogenesis of AD and generates Ca2+ influx, generation of reactive oxygen species, secondary excitotoxicity, and activation of caspases such as caspase 3. Here we demonstrate that Aβ42-induced cell death is accompanied by the release of cytochrome *c* from mitochondria, the upstream signaling component of caspases activation and that etazolate significantly inhibits Aβ-induced cytochrome *c* release. The nearly complete reversal effect of etazolate effects on neuroprotection against Aβ42 and Aβ25–35 peptides by $GABA_A$ receptors antagonists picrotoxin (PTX), gabazine/SR95531 and bicuculine strongly indicates that this neuroprotective property is mediated through $GABA_A$ receptors. Indeed, etazolate binds to $GABA_A$ but not $GABA_B$ receptors. Interestingly, a number of different classes of molecules acting at the GABA receptor such as the agonists GABA, muscimol, taurine, phenobarbital, carbamazepine and valproic acid as well as the $GABA_A$ antagonist D-securinine, can attenuate Aβ-induced neurotoxicity in a bicuculine and picrotoxine sensitive manner (Gu *et al.* 2003; Louzada *et al.* 2004; Lin and Jun-Tian 2004). In contrast, we and others (Lee *et al.* 2005) show that baclofen, a $GABA_B$ agonist, does not exhibit any protective effect against Aβ, indicating a $GABA_A$ receptor specific subtype requirement for neuroprotection. Since $GABA_A$ receptors mediate the influx of Cl- in mature neurons such as the ones used in this study, and Aβ has been reported to increase neuronal excitability by inhibiting GABA-induced Cl- current in neurons (Lee *et al.* 2005), it can be envisioned that the protective effect of etazolate may be associated with a normalization of Cl- flux mediated by the $GABA_A$ receptor.

Etazolate modulates the $GABA_A$ receptor pharmacology in a subunit-selective manner with IC50 around 1 μM (Thompson *et al.* 2002). However, it should be noted that etazolate is also known to have a phosphodiesterase 4 (PDE4) inhibitor activity (Wang *et al.* 1997). By stimulating cAMP levels, the PDE4 inhibitor Rolipram and the PDE4-CREB pathway show beneficial effect at counteracting Aβ-induced memory, synaptic and dendritic alterations (Gong *et al.* 2004; Shrestha *et al.* 2006). However, Rolipram exerts its beneficial effects in AD mouse models on cognition and synaptic functions independently of Aβ (Comery *et al.* 2005) and does not induce sAPPα nor prevent Aβ–mediated neurodegeneration *in vitro* (our unpublished observations). Interestingly, cAMP functionally modulates $GABA_A$ receptor (Moss *et al.* 1992) and has been linked to sAPPα release (Maillet *et al.* 2003). Therefore, we cannot exclude a convergence of the two pathways and the possibility that some of the effects shown here by etazolate are also mediated through elevated cAMP levels in cortical neurons. However, the finding that three $GABA_A$ receptor blockers, bicuculine, picrotoxine and gabazine, prevent the neuroprotection exerted by etazolate against Aβ–induced neurotoxicity

clearly implicates the $GABA_A$ receptor in etazolate–mediated, sAPPα–dependent, neuroprotection.

Irrespective of the mechanism, etazolate is likely to offer the benefit of being neuroprotective and plaque-reducing agent *in vivo* because stimulation of the α-secretase activity may reduce Aβ formation. Using a rat Aβ40 specific ELISA and previously described β-secretase and γ-secretase assays (Désiré *et al.* 2005), we show here that etazolate does not directly impact the amyloidogenic pathway. Our *in vivo* and *in vitro* data thus suggest a selective effect on the α-secretase pathway. Guinea pigs treated with etazolate, however, exhibit a 20% reduction in total (soluble and insoluble) Aβ40 quantified by ELISA, when compared to control (data not shown). This may indicate that etazolate acts to redirect the processing of APP towards the α-secretase pathway *in vivo*. It has been demonstrated that, *in vivo*, even subtle enhancement of α-secretase activity (30% changes) can ultimately result in two fold or more changes in Aβ levels and can lead to almost complete prevention of amyloid plaque formation and alleviate cognitive deficits (Postina *et al.* 2004). This allows us to anticipate that a significant reduction of Aβ production after long-term activation of the α-secretase pathway by etazolate may be achieved in AD models or AD patient, allowing amyloid plaque reduction and cognitive benefit.

Drugs acting at the $GABA_A$ receptor complex are of potential use in the treatment of memory disorders (Moran *et al.* 1992). Etazolate also presents procognitive properties such as the ability to prevent scopolamine-induced amnesia in rats (our unpublished observations). Interestingly, the deleterious effect of scopolamine on memory can be counteracted by sAPPα (Meziane *et al.* 1998). Therefore, etazolate presents the ability to link $GABA_A$ and non-amyloidogenic α- secretase pathways and both these pathways make it likely that etazolate treatment will also result in procognitive properties in AD. Interestingly, sAPPα release is coupled to neuronal electrical state and synaptic activity and ADAM-10 trafficking and activity is dependent on synaptic activity (Kamenetz *et al.* 2003; Marcello *et al.* 2007). In fact, indirect evidences exist to suggest that molecules acting at the GABA system can modulate the activity of metalloproteases from the ADAM family *in vivo* (Shastry *et al.* 2006). If this was demonstrated in CNS, GABAergic modulators may offer an unexpected new avenue for the development of AD through the control of the non-amyloidogenic α-secretase pathway.

In transgenic hAPP mice that develop AD-like abnormalities including cognitive deficits, Palop *et al.* (2007) have shown that Aβ-induced neurological deficits may involve aberrant increases in neuronal excitability and compensatory inhibitory mechanisms such as GABAergic function. Thus, modulation of Aβ-induced neuronal overexcitation by etazolate may prevent the development of AD-related neurological deficits and more studies are needed to determine how etazolate synaptic impact can be reinforced via sAPPα for cognitive benefit.

Finally, the pharmacokinetic and safety profiles of etazolate have been evaluated in Phase 1 studies which have established that etazolate is a well tolerated drug devoid of side effects (data not shown). Therefore, etazolate is an attractive candidate for the treatment of AD which may offer novel symptomatic and disease modifying opportunities.

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PRESS RELEASE

ExonHit announces last patient out for EHT 0202 Phase IIa study in Alzheimer

- Top-line EHT 0202 data to be presented in Florence on September 14th

Paris, France – June 16, 2009 – ExonHit Therapeutics (Alternext: ALEHT) today announced that clinical testing of EHT 0202, its lead therapeutic compound in Alzheimer's disease, is progressing well. Final patient dosing for the Phase IIa proof-of-concept clinical trial assessing EHT 0202 in patients with Alzheimer's disease is completed.

"In light of the neuroprotective and procognitive effects demonstrated by EHT 0202 in preclinical studies, we believe that our drug candidate has the potential to modify the course of Alzheimer's disease and might open a new era in the treatment of this devastating disease," commented Dr. Loïc Maurel, President of the Management Board of ExonHit Therapeutics. "We look forward to the outcome of this Phase IIa trial and are planning to initiate out-licensing discussions with potential partners next fall."

Top-line data from the EHT 0202/002 Phase IIa study will be presented at the 13th Congress of the European Federation of Neurological Societies on September 14th in Florence, Italy.

The study was conducted in 23 centers across France under the supervision of Professor Bruno Vellas, Head of Alzheimer's Disease Clinical Research Center and Gerontopole, Toulouse University Hospital, France. A total of 197 ambulatory patients suffering from mild to moderate Alzheimer's disease were selected and 158 of them were randomized to receive oral study treatment over a three-month period.

This multicenter, randomized, double-blind, placebo-controlled study was designed to assess the safety and tolerability, as a primary objective, and also exploratory efficacy of EHT 0202 in patients with Alzheimer's disease. The effect of two different doses of EHT 0202 (either 40 or 80 mg twice a day) as adjunctive therapy to one acetylcholinesterase inhibitor is evaluated in comparison to placebo. Efficacy is evaluated on multiple parameters by using different scales, including a battery of cognitive assessment tests (ADAS-Cog, NTB, MMSE), assessment of patients' daily living activities, and also global behavioural assessment.

About EHT 0202

EHT 0202 has a novel mechanism of action when compared to existing Alzheimer's disease therapeutics: it stimulates the α-secretase pathway, thus enhancing the production of the procognitive and neuroprotective sAPPα fragment of APP (Amyloid Precursor Protein). The stimulation of the α-secretase pathway being to the detriment of Aβ amyloid peptide production, EHT 0202 potentially reduces toxic Aβ plaque formation (1).

Phase I studies demonstrated good tolerability of EHT 0202 in both young and aged healthy volunteers; importantly, no sedation was observed clinically.

Preclinical studies have shown that EHT 0202 protects cortical neurons against Aβ42-induced stress and that this neuroprotection is associated with sAPPα induction. EHT 0202 has also demonstrated pro-cognitive properties in several animal models: age-related memory impairment and scopolamine-induced amnesia (2).

About Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative condition that is the most frequent cause of dementia in the aging population. An estimated 26.6 million people worldwide had Alzheimer in 2006. This number is anticipated to quadruple by 2050 to more than 100 million; 1 in 85 persons worldwide will be living with the disease (3). In France alone, 800,000 people, or 18% of people above 75 years old, have Alzheimer's disease (4).

About ExonHit Therapeutics

ExonHit Therapeutics (Alternext: ALEHT) is a fast emerging healthcare player active in both therapeutics and diagnostics. The Company is applying its proprietary technology, based on the analysis of alternative RNA splicing, to develop innovative blood based diagnostic tests and therapeutics for neurodegenerative and cancer indications. ExonHit has a balanced investment strategy with internal development programs and strategic collaborations, in particular with bioMérieux and Allergan.

ExonHit is headquartered in Paris, France and has U.S. offices in Gaithersburg, Maryland. The Company is listed on Alternext of NYSE Euronext Paris. For more information, please visit http://www.exonhit.com.

Disclaimer

This press release contains elements that are not historical facts including, without limitation, certain statements on future expectations and other forward-looking statements. Such statements are based on management's current views and assumptions and involve known and unknown risks and uncertainties that could cause actual results, performance or events to differ materially from those anticipated.

In addition, ExonHit Therapeutics, its shareholders, and its affiliates, directors, officers, advisors and employees have not verified the accuracy of, and make no representations or warranties in relation to, statistical data or predictions contained in this press release that were taken or derived from third party sources or industry publications, and such statistical data and predictions are used in this press release for information purposes only.

Finally, this press release may be drafted in the French and English languages. In an event of differences between the texts, the French language version shall prevail.

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